Standardization of Molecular Biomarker Assays from Discovery and Development to the Clinical Laboratory: Lessons Learned

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Standards of Clinical Laboratory Testing The Basics

What's CLIA?

Clinical Laboratory Improvement Amendments

- Congress passed the Clinical Laboratory Improvement Amendments in 1988 which addresses good laboratory practice by ***establishing quality standards for all laboratory testing*** to ensure accuracy, reliability and timeliness ***for patient test results*** regardless of where the test was performed.
- A laboratory is any facility that does laboratory testing on specimens derived from humans to give information for the diagnosis, prevention, treatment of disease, or impairment of, or assessment of health.
- CMS regulates all laboratory testing through CLIA.
- CLIA does NOT address clinical utility, test sensitivity, result interpretation.

College of American Pathologists (CAP)

- World's largest association composed exclusively of pathologists
- Leader in laboratory quality assurance
- CAP laboratory accreditation program originated in the 1960s
- Recognized by the federal government as being equal to or more stringent than the government's own inspection program
- Deemed status with CLIA
- CAP accreditation by the Commission on Laboratory Accreditation of the CAP based on a stringent on site inspection every two years which includes examination of:
  - Standard operating procedures/laboratory records
  - Quality control procedures/quality assurance programs
  - Qualification of directors and staff
  - Laboratory equipment
  - Facilities
  - Safety Program
  - Overall laboratory management
- Deficiency citations
  - Phase I: Do not seriously affect quality of patient care or endanger lab worker. (Written response required)
  - Phase II: May seriously affect quality of patient care or health/safety of hospital/lab personnel. (Requires corrective plan/support documentation of plan implementation)

Compliance with CLIA/CAP SOPs

- Specimen Handling/Specimen Rejection Criteria/Specimen Storage
- Principle/Procedure
- Equipment
- Supplies
- Controls/Reagents/Reagent Preparation
- Quality Control/Criteria for Controls
- Interpretation and Procedure Limitations
- Troubleshooting Guide
- References

Molecular Assays

Clinical Laboratory Definitions/Requirements

CAP Checklist (CAP-CKL)

The Basics

Molecular Assay Validation Definitions CAP-CKL

- Analytical performance characteristics of the assay must be verified/ established in accordance with Test Method Validation requirements in CAP-CKL including:
  - Analytic sensitivity: ability of an assay to detect a given analyte (i.e. the lower limit of detection).
  - Analytic specificity: false-positive rate in addition to the degree to which interfering substances are not detected by an assay.
  - Other analytic parameters: calibration procedures, materials, verification criteria and other performance characteristics such as linearity, specimen stability or carryover.
  - Diagnostic sensitivity: ability of an assay to detect a disease or clinical condition.
  - Clinical Specificity: degree to which an assay is negative when disease is absent.
  - Diagnostic sensitivity and specificity must be determined in a "blinded" fashion and relative to some "gold standard" (e.g. biopsy findings, clinical findings, etc.).
    - TP = true positive - TN = true negative
    - FN = false negative - FP = false positive
  - Laboratory-developed test (LDT)
    - Test performed by clinical laboratory where test was developed.
    - Test neither FDA-cleared nor FDA-approved or an FDA-cleared/approved test modified by the laboratory.

Clinical Laboratory Phase I/II
CAP-CKL Selected Requirements
Molecular Assay Validation

- Validation Studies – LDTs or Modified FDA-Approved/Cleared Tests (Phase II)
  - Laboratory documentation performed analytic validation studies to establish performance characteristics (accuracy, precision, reportable range, reference range, analytic sensitivity, and analytic specificity).
- Validation Studies – Specimen Selection and Types (Phase II)
- Validation Study Comparison (Phase II)
  - Each validation study compared to another valid assay.
- Reference/Reportable Range – Qualitative and Quantitative (Phase II)
  - Reference and reportable ranges are defined.
- Clinical Performance Characteristics (Phase II)
  - Documented per literature citations or internal study results.
- LDT Reporting (Phase II)
  - Description of method, statement assay developed by laboratory, and appropriate performance characteristics.
- LDT Clinical Claims Validation (Phase II)
  - Any clinical claims regarding laboratory-developed tests validated (i.e. sensitivity, specificity, risk prediction, clinical usefulness, etc.

Clinical Laboratory Phase I/II CAP-CKL
Selected Requirements
Molecular Assay Collection and Preparation

- Neoplastic Cell Content (Phase II)
  - For paraffin embedded tumor specimens, there is documentation of histological assessment of neoplastic cell content.
- Nucleic Acid Extraction/Isolation/Purification (Phase II) (documentation/validated method in-house)
- Nucleic Acid Quantity (measured when appropriate) (Phase II)
- Nucleic Acid Quality (integrity/purity assessed when appropriate)(Phase II)
  - Appropriate "housekeeping" mRNA internal control for RNA integrity.
- Ribonuclease-Free Conditions (assays that detect RNA or use RNA probe) (Phase I)
- Concentration Techniques verified for quantitative tests. (Phase I)
- Specimen Storage and Retention (prompt retrieval for further testing) (Phase II)

Clinical Laboratory Phase I/II CAP-CKL Selected Requirements

Amplification

- Probe Characteristics (Phase II)
  - Documented information on nature of probe or primer to permit interpretation/troubleshooting of test results
- Carryover – PCR (Phase II)
  - Physical containment and procedural controls to minimize false positives.
- Internal Controls – Nucleic Acid Amplification (Phase II)
  - All internal controls to detect false negative reaction secondary to extraction failure or presence of inhibitor.
- Calibration Procedures (Phase II)
  - Adequate and documented
- Calibration Materials (Phase II)
  - High quality/appropriate for target values

Clinical Laboratory Phase I/II CAP-CKL Selected Requirements

Sequencing

- Gene Information (Phase II)
  - Wild type sequence, reported mutations, polymorphisms.
- Sequencing Assay Optimization (Phase II)
  - Ensure readable signal throughout the length of target region, ready detection of sequence variants, especially heterozygous state.
- Sequencing Data Criteria (Phase I)
  - Established for acceptance/interpretation of primary sequencing data
- Process Documentation (Phase I)
  - Analytical wet bench process to generate next generation sequencing data, e.g. sample, library preparation, sequence generation.

SOP for Validation New Molecular Tests at UMB-BRL Molecular Diagnostics Laboratory

- Number of samples for validation represents a typical test run size (e.g. one month test volume, usually 20 – 50 samples).
- Validation of specific target amplification by direct sequencing of the product for PCR-based assays.
- 100% result concordance desired for validation. Discordant samples are investigated to determine the reason (e.g. sample source problem, low viral copy, clinical profile, etc.).
- Blind studies test proficiency of molecular diagnostics laboratory.
- Whenever possible, samples from a CAP certified laboratory.
- After initial validation, Proficiency Testing (PT) minimum 2x/yr with CAP survey samples.
- Test w/o CAP PT, define alternative method for verification minimum 2x/yr.
- Sufficient information regarding nature of probe or primer to permit interpretation/troubleshooting of test.

SOP for Validation New Molecular Tests at UMB-BRL Molecular Diagnostics Laboratory

- Document items of importance including:
  - Type and origin probe or sequence
  - Oligonucleotide sequence and complementary sequence or gene region recognized
  - Appropriate restriction enzyme map of the DNA
  - Known polymorphisms
  - Sites resistant to endonuclease digestion and cross-hybridizing bands
  - Labeling methods used
  - Standards for adequacy of hybridization of amplification
  - Validation documentation available for the period procedure is used in laboratory, or two years, whichever is longer.

SOP for Validation New Molecular Tests at UMB-BRL Molecular Diagnostics Laboratory

- Document items of importance including:
  - Qualitative and screening tests – determine/document basis for specifying reportable results as positive, negative, or degree of reactivity.
  - Periodic evaluation of appropriateness of reference ranges, and corrective actions, if necessary.
  - Changes in analytic methodology so test results and interpretations significantly different must be explained to users.
  - Laboratory performing same test using different methodologies/ instruments, or same test at multiple sites, must evaluate/define relationship between test results minimum 2x/yr.
  - Training checklists/competency for appropriate number of techs based on estimated test volumes.
  - If laboratory relocates/changes testing sites, document performance specifications for each test method.

Molecular Biomarker Assay Validation Lessons Learned

A Case Study

MSA Assay Validation for Detection of Bladder Cancer SOP/Qualification

UMB BRL Responsibility

- UMB-BRL (CLIA/CAP certified) served as a reference laboratory for QA of the MSA test performed by Commonwealth Biotechnologies, Inc. (CBI) (CLIA accredited / GLP compliant)
- Only perform the MSA test
- Goal is to provide quality assurance for 10% of the paired blood and urine study samples
- All testing is blinded

UMB- BRL Responsibility (cont'd)

- The assay consists of STR PCR analysis of 15 previously validated microsatellite markers located within 14 gene loci *(D4S243, D21S1245, FGA, D17S695, D16S476, D9S171, IFN-A, D20S48, D17S654, D16S310, TH01, D9S162, D9S747, MBP and MBPa)*
- Expected assay sensitivity is 83-95% and specificity is 100% for normal versus abnormal determination of the sample pair

Qualification

- Prior to validation 5 rounds of qualification testing were performed
- Testing consisted of previously extracted DNA from blood and urine sample pairs selected and blinded by JHC
- The blinded sample sets were split by CBI who shipped the DNA aliquots to UMB-BRL
- Sample sets were then jointly assayed by CBI and UMB-BRL to assess and assure concordance
- Data analysis of the blinded results was performed by DMCC

Qualification Rounds 1 and 2

Round	Samples	Concordance	Issues uncovered
1	7 pairs of matched Blood/Urine (B/U) DNA	78%	Difference in Primer pairs used
2	20 pairs matched B/U DNA from ineligible participants in the MSA study	73%	▪ Need for new Pos control ▪ Clear acceptance criteria for alleles (ie Peak Height) and repeat reactions ▪ Need for data result spreadsheet for reporting of data

Qualification Rounds 3 and 4

Round	Samples	Concordance	Issues uncovered
3	22 pairs matched B/U DNA from ineligible participants in the MSA study	87%	Refinement of SOP with regard to interpretation and acceptance criteria
4	20 pairs matched B/U DNA from ineligible participants in the MSA study	73%	▪Used new reagents (new PCR master mix, HLPC primers ▪Peak heights too high!!!

Fifth MSA Qualification Round Results

- Prior to Round 5, performed unblinded study between UMMB and CBI to determine optimum electrophoresis conditions.
- For Round 5, 20 blinded matched pairs were performed using new instrument conditions
- Concordance was 92%; the concordance for loci that were evaluable for both laboratories was 96%. The overall evaluation of the samples (positive or negative for MSI) was identical (20/20).

Post Fifth Qualification Round additional QC comparisons and test validation performed.

MSA Study
Lessons Learned and Recommendations for Future Clinical Assay Validation

- Involve QA laboratory early in the process of the SOP development and <u>data interpretation</u>
- Perform unblinded parallel studies during assay development to assess efficacy of all assay parameters:
  - Instrumentation (e.g. comparative calibration)
  - Reagents (e.g. handling, storage)
  - General assay performance and set up (technical assessment)
- Criteria for assay parameters and date interpretation
- Clearly define interpretation guidelines that result in level of consistency between laboratories

MSA Study
Lessons Learned and Recommendations for Future Clinical Assay Validation (Cont'd)

- Clearly define all aspects of these assay parameters in the SOP
- Continued parallel and proficiency testing as BDL develops and evaluates biomarker(s).
- Incorporate well defined quality assurance oversight/methods to provide more consistent results throughout the development/validation.

MiRNA Assay

Discovery Laboratory to Clinical Laboratory

Major Techniques used for Detection of miRNAs

- Microarray
  - High throughput technique
  - Low sensitivity
  - Expensive
  - Results need to be validated by other methods (e.g. qRT-PCR or *In situ* hybridization
- Deep-sequencing
  - Provides more information on miRNA aberrations
  - Expensive
  - Time consuming

Major Techniques used for Detection of miRNAs (Cont'd)

- *In situ hybridization*
  - Time consuming
  - Non-quantitative
  - Low throughput
- <u>qRT-PCR assay</u>
  - Extensive experience in CLIA/CAP laboratories for clinical specimens
  - Flexible
  - Cost effective
  - Easily performed

Summary

- UMB-BRL established a standardized protocol between the discovery/ development laboratory and the clinical laboratory (CLIA/CAP) to assay miRNA in cell lines.
- Develop guidelines for consistent inter-laboratory result interpretation.
- Creating a set of NIST standards for the qualitative and quantitative assessment of assay performance and to ascertain clinical usefulness of miRNAs in early detection of stage 1 non-small cell lung cancer in sputum and bronchial washings.

Application of a vimentin gene methylation assay(s) as a potential biomarker for colon cancer

(Case Western Reserve University: Sandy Markowitz, MD, PhD, University of Michigan: Dean Brenner, MD, UMB BRL: Sanford Stass, MD, Debra KuKuruga, PhD)

- Aberrant methylation of exon-1 sequences within the non-transcribed vimentin gene is a novel molecular biomarker of colon cancer and can be successfully detected in fecal DNA. Aberrant vimentin gene methylation in colon cancer patients has been previously reported with a sensitivity of approximately 50% and a specificity of 90%.
- Captured fecal DNA will be assayed for vimentin gene methylation as a biomarker for early detection of colon cancer
- UMB BRL will serve as a CLIA/CAP accredited reference laboratory for vimentin assay(s) and receive aliquots of stool and provide quantitative measurements of methylated vimentin DNA
- Real time PCR SOP being developed for transfer from Dr. Markowitz's research laboratory to UMB-BRL.

Vimentin Assay
Selected Issues in Transfer from Discovery/ Developmental Laboratory to Clinical Laboratory

- Buffer effects sensitivity - using Roche buffer (Essential) vs. ABI Taqman universal buffer effects the sensitivity of the assay, i.e. Ct values are higher (high 30's to 40) vs. low to mid-30's with Roche buffer.
- Presence or absence of ROX (florescent dye) may effect efficiency of PCR-assay more sensitive without ROX.
- Parameters for performing standard curve
  - Diluted positive control after bisulfate treatment
  - Add irrelevant carrier nucleic acid MS2RNA (blocks nonspecific nucleic acid binding sites – used successfully in miRNA procedure) to extracting buffer before dilution assists in curve linearity at higher dilutions.
- HPLC purified primers.

Standardization of Molecular Biomarker Assays from Discovery/Development to the Clinical Laboratory: Lessons Learned

Conclusion

Standardization of Molecular Biomarker Assays from Discovery/Development to the Clinical Laboratory: Lessons Learned

Conclusion

- Developmental laboratories generally use assays for a long time prior to discovery/development of biomarker. SOPs may have been modified on an ongoing basis so difficult to evaluate assay efficacy.
- Early identification by the BRL of promising platform(s)/ procedures used by BDL so BRLs prepared for technology transfer.
- Assays or equipment used by BDLs are not in use in a BRL therefore delays while BRLs start from the beginning to learn/optimize/ increase reliability of assays.

Standardization of Molecular Biomarker Assays from Discovery/Development to the Clinical Laboratory: Lessons Learned

Conclusion

- Lack of appropriate SOPs
  - SOP changes w/o stringent review/documentation
  - No documented rationale to support SOP changes
- Appropriate QC
  - Lack of equipment calibration, QC, maintenance or monitoring, including pipet QC, storage conditions, etc.
  - The same model of instrument/platform may not function the same and produce comparable results between BDL and BRL (calibration, SOP, etc.)
  - Lack of reagent QC including source, tracking by lot, shipping number, storage conditions; HPLC grade not used
  - Kits used for testing not at clinical level (e.g. kits purchased outside U.S. may not meet clinical standards) and contents mixed

Standardization of Molecular Biomarker Assays from Discovery/Development to the Clinical Laboratory: Lessons Learned

Conclusion

- Appropriate QC (cont'd)
  - Lack of controls in assay
  - QC of in-house reagents and lack of expiration dates
- Budget comparisons between BDL and BRL different since not all testing components included (e.g. standard curves, controls, etc.)
- Need improved communication between BDL and BRL as to goals
- Alternate methods used in the BDL for the same assay by different technologists or same assay performed differently by technologists in BDL makes it difficult for BRL to develop CLIA/CAP SOP.
- Lack of clarity on how BDL calculates results
- Specimen reagents not stored at proper temperatures; temperatures of refrigerators/freezers not recorded.
- Improve documentation specimen labeling and tracking (unique identifiers)
- Staff training - Competency
  - No documentation of competency training
  - Inconsistencies in running an assay from batch to batch
  - Staff follows procedure as delineated in SOP
  - Different staff "do it their own way"

Selected Recommendations to Facilitate Translation of Assays from Discovery/Development to Clinical Laboratories

Selected Recommendations to Facilitate Translation of Assays from Discovery/Development to Clinical Laboratories

- Very early in the process discovery/development laboratory partners with clinical laboratory to discuss assay development and implementation.
- Proficiency and parallel testing for discovery/ development laboratory.
- Oversight through a translational research laboratory (CLIA/CAP accredited).

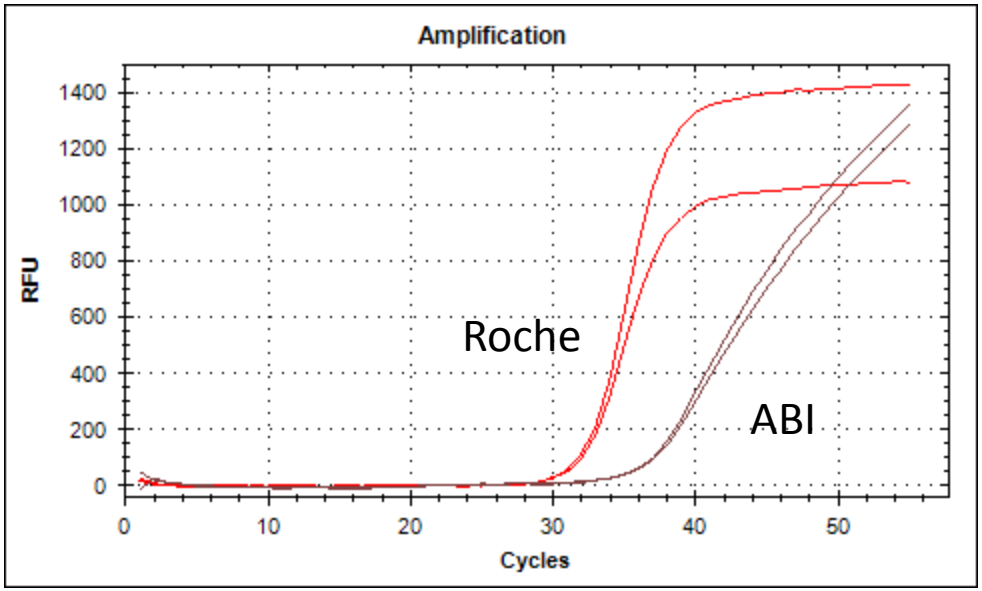
Translational Genomics Laboratory

- Established April, 2011 at University of Maryland School of Medicine
- Directors-Department of Pathology and designated clinical investigators.
- Fully accredited by CLIA (#21D2027356; exp. 6/25/2014)
- Bridge from clinical research University of Maryland School of Medicine to CLIA/CAP clinical laboratory at University of Maryland Medical Center.
- Provides genomic tools for translational research and clinical trials.
- Services to include mutational analysis by genotyping and DNA sequencing, gene expression analysis, and targeted sequencing by next-generation sequencing methods.

Require Proficiency Testing For Discovery and Developmental Laboratories

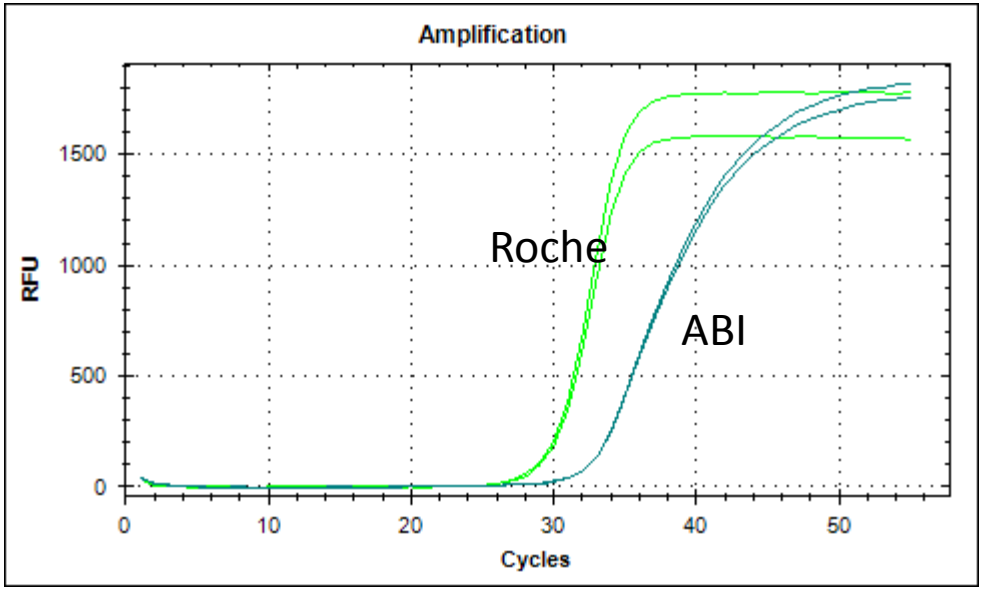
- Proficiency Testing (PT) – Determination of laboratory testing performance by interlaboratory comparisons
  - PT program periodically sends multiple specimens to a group of laboratories for analysis and/or identification
  - Program compares each laboratory's results with those of other laboratories in the group and/or with an assigned value
- Alternative assessment - Determination of laboratory testing performance by means other than PT (i.e. split-sample testing, testing by a different method, etc.)

Vimentin Methlylated BioRad Instrument (Roche vs ABI Buffer)



Note that on BioRad machine
The curves span 3 logs of signal,
While on ABI machines it only
Spans 1 log.

Vimentin Unmethlyated BioRad Instrument (Roche vs ABI Buffer)



Require Proficiency Testing For Discovery and Developmental Laboratories

A Proposal

Require Proficiency Testing For Discovery and Developmental Laboratories

- PT - blinded samples must be performed and measured as if they were from patients.
- Typically PT is performed 2-3 times/year. During each proficiency challenge usually 5 separate samples, may be as few as 3, on which the proficiency test performed and reported to the reference laboratory.
- The reference laboratory with demonstrated competency (determines reference value) then provides feedback on the testing laboratory's performance. Typically acceptable performance is 80% of the results from the samples are within the acceptable result range.

Proficiency Testing

- PT validates one's measurement processes
  - Measurement method
  - Technical training
  - Traceability of standards
  - Calibration
  - Corrective/preventive actions from PT for unacceptable results
  - No interlaboratory communication
  - No referral to another laboratory

Molecular Assays
A Future Challenge

Next Generation Sequencing

CDC – Division of Laboratory Science and Standards
Next Generation Sequencing
Translation from Research to Clinical Applications

- **Next-generation sequencing** (NGS) has **expanded beyond research applications** to **deliver clinically actionable test results** that can effectively inform medical decision making.
- This new area of clinical **testing lacks uniform practices for quality assurance (QA) and quality control (QC)** that are essential to ensure the analytical validity of test results.
- Areas of focus
  - Emphasis on heritable sequence variations
  - Identification and application of quality metrics that are platform independent
  - Focus on test validation, QC, reference materials (RMs), proficiency testing (PT) and alternate assessment (AA)

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Next Generation Sequencing
Translation from Research to Clinical Applications

Steps of NGS that Require Test Standardization, Validation and QA/QC

- Sample Preparation
- Library Preparation
- Sequence Generation
- Sequence Analysis
- Result Reporting

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Next Generation Sequencing
Translation from Research to Clinical Applications

Test Validation

- For NGS, validation is the process of establishing analytical performance specifications and determining QC practices needed for future patient testing within the laboratory in which the test is performed.
- The complexity of NGS requires consideration for both platform and rest specific validation processes.

Platform Validation

To establish an acceptable error rate in the sequencing of a reference material for each NGS technology and informatics analysis tool.

Test Validation

To establish an acceptable error rate for detection of specific targeted sequence variants for each test's technology and informatics data analysis.

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Next Generation Sequencing
Translation from Research to Clinical Applications

Performance Characteristics

	Definitions for NGS applications	Metrics and processes of NGS analytic performance
Accuracy	Closeness of agreement between a measured value and the true value, which for NGS is the accepted reference sequence.	Coverage – The number of independent overlapping base calls made at a given position. - Depth of coverage - Average coverage - Uniformity or distribution of coverage Quality scores – Confidence in a base or variant call.
Precision	The degree to which repeated measurements give the same result (repeatability and reproducibility).	Monitor performance for: - Library variability: independent library preparations - Intra-run variability: same sample, same library, same run. - Inter-run variability: same sample, same library, different runs. - Inter-operator variability.

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Translation from Research to Clinical Applications

Performance Characteristics

	Definitions for NGS applications	Metrics and processes of NGS analytic performance
Analytic Sensitivity	Likelihood that the assay will detect a sequence variation, if present.	• Depth of coverage must be sufficient to minimize a loss of sensitivity and specificity. • The depth of coverage achieved with NGS will vary across the genome and therefore should be established across all regions of the sequence targeted for the clinical application.
Analytic Specificity	Probability that the assay will not detect a sequence variation, if not present.	

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Performance Characteristics

	Definitions for NGS applications	Metrics and processes of NGS analytic performance
Reportable Range	The regions of the genome for which the NGS technology can accurately produce sequence information (e.g. multiple genes, exome, large genomic regions).	Define areas of difficulty (e.g. repeat regions, insertions and deletions, allele dropouts) near the regions of interest.
Reference Range	Establishment of reportable sequence variations expected to occur in the target population that the assay can detect.	Materials containing the type of sequence variation(s) appropriately distributed within the target sequence may establish the capacity of the test to detect similar disease-associated mutations.

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Next Generation Sequencing
Translation from Research to Clinical Applications

QC/QA Considerations

- Monitoring established performance specifications
  - Coverage
  - Quality Scores
  - Allelic Read Percentage
  - Mapping Quality
  - Strand bias
  - GC Skew
  - Decline in Signal Intensity
- Use reference materials to monitor assay performance and for PT/AA
  - Reference materials are needed that contain the range and distribution of sequence variations comparable to those which the assay is designed to detect.
  - Method-based PT may be a component of an inter-laboratory comparison because of the size of the genome interrogated and the number of potential sequence variants targeted.
  - PT/AA Materials useful for NGS:
    - DNA from a well characterized cell line or patient sample (PT)
    - Electronic data (PT)
    - Inter-laboratory sample or electronic data exchange (AA)

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Next Generation Sequencing
Translation from Research to Clinical Applications

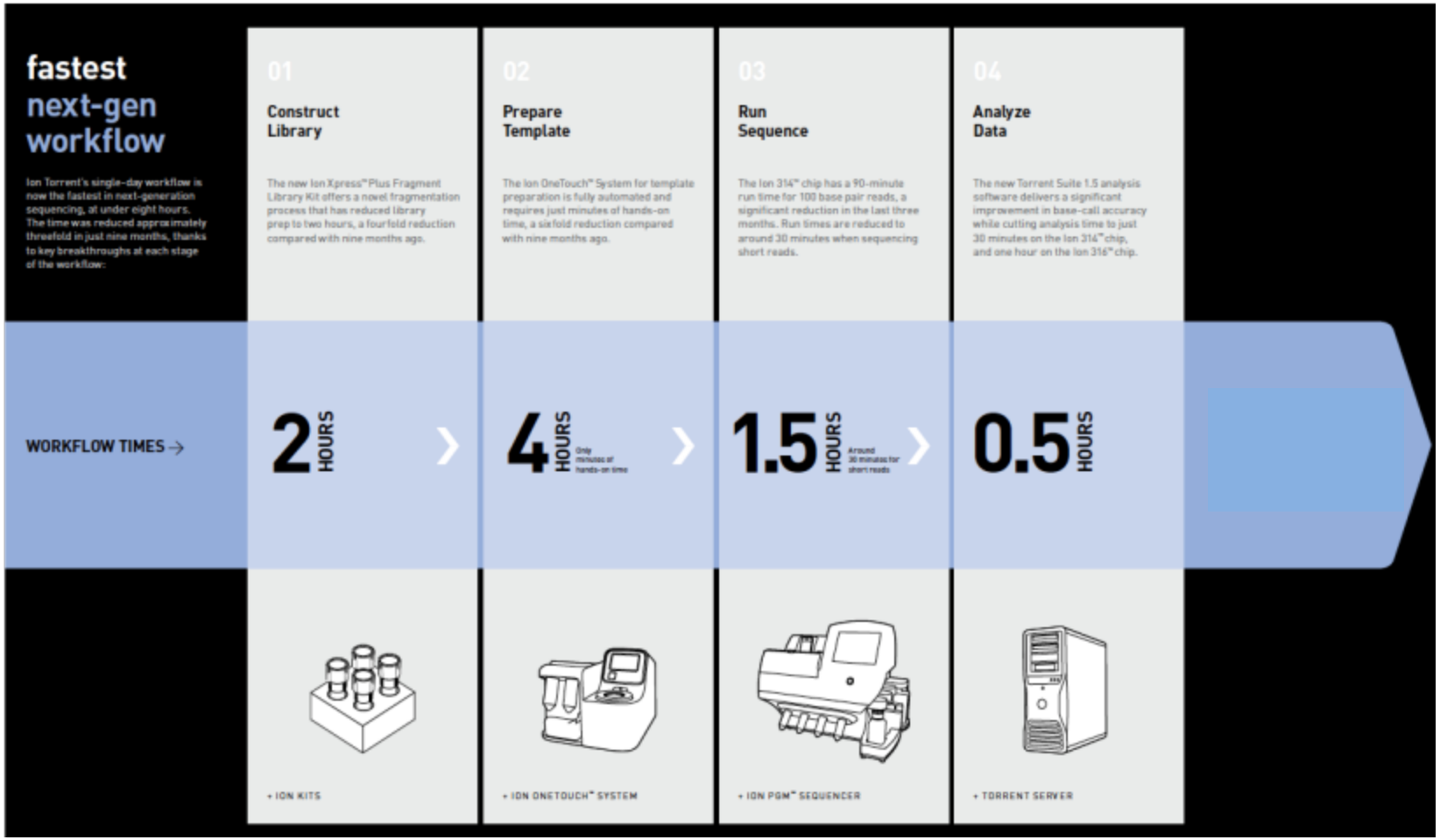
Conclusions

- Validation should establish a depth of coverage sufficient to minimize a loss of sensitivity and specificity.
- Method-based PT challenges should take into account that different laboratories will interrogate different regions of the genome.
- Reference materials and sequences used for test validation, QA/QC and PT/AA should include the types of sequence variants targeted by the test and appropriately distributed across the targeted region of the genome. This is in addition to use of actual patient samples that would be expected to have only a subset of the targeted sequence variants.

Ion Torrent Sequencing Technology and Cancer Research

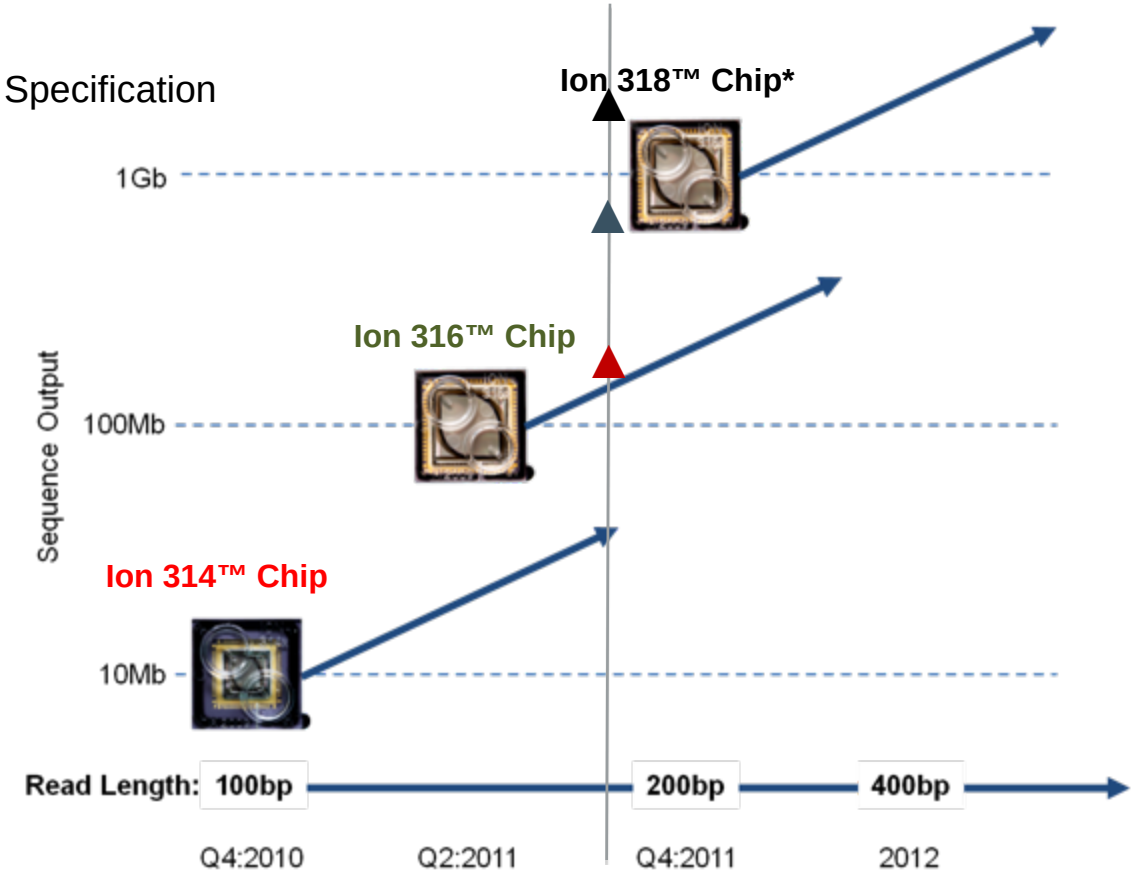
Speed

Fastest Next-Gen Workflow



Semiconductor Scalability – 100X in the First Year

10Mb to 1Gb in one year (100x) — The Chip is the Machine™



Dec 2011 R&D performance

- ▲ Ion 318 >1400 Mb
- ▲ Ion 316 >850 Mb
- ▲ Ion 314 >150 Mb

*Some products have not yet been officially released and information about those products is subject to change without notice
Sept 2011 data for 314 and 316 chips based on internal R&D runs

Validation of Somatic Mutations

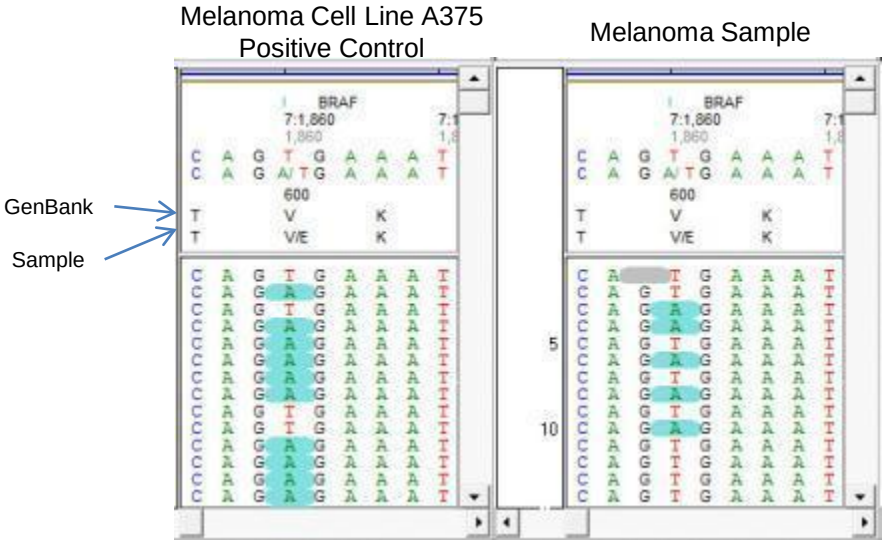
Barcoded amplicon sequencing for detection of BRAF & NRAS mutations in melanomas

Demonstrated sequencing is possible from 6 year old FFPE tissue.

53 samples sequenced representing 47 unique samples on four 314 chips

Positive controls 100% accurate and

Mutations found in 47% of samples



Dr. George Watts

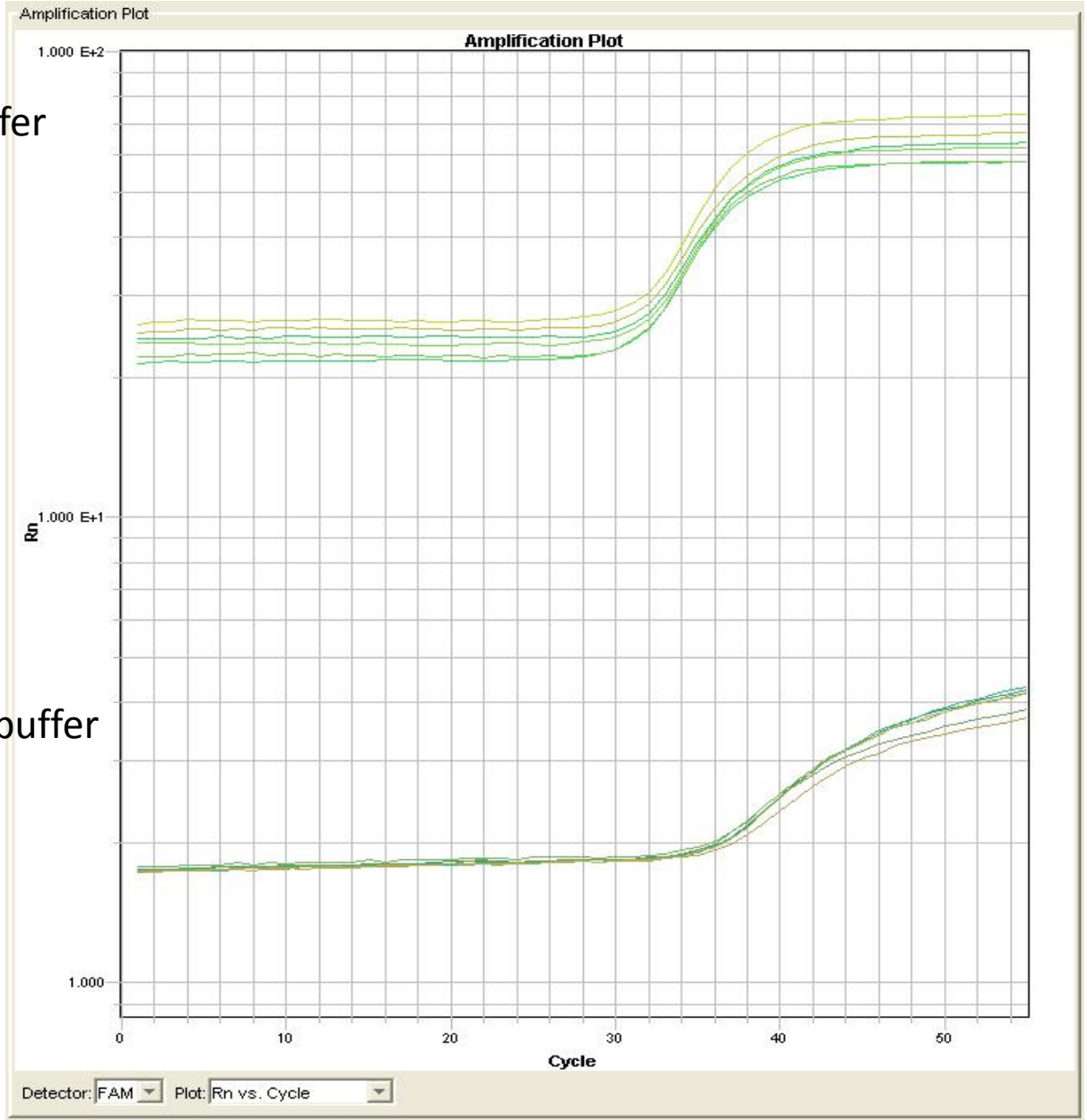
Ion AmpliSeq™ Cancer Panel:

KRAS	BRAF	EGFR	TP53	PIK3CA	CSF1R	JAK2
NRAS	PTPN11	ERBB2	SRC	FGFR3	NPM1	CDKN2A
RET	HNF1A	SMAD4	GNAS	PDGFRA	MPL	ABL1
PTEN	FLT3	STK11	SMARCB1	KIT	MET	NOTCH1
FGFR2	RB1	JAK3	VHL	KDR	SMO	
HRAS	AKT1	ALK	MLH1	FBXW7	ERBB4	
ATM	CDH1	IDH1	CTNNB1	APC	FGFR1	

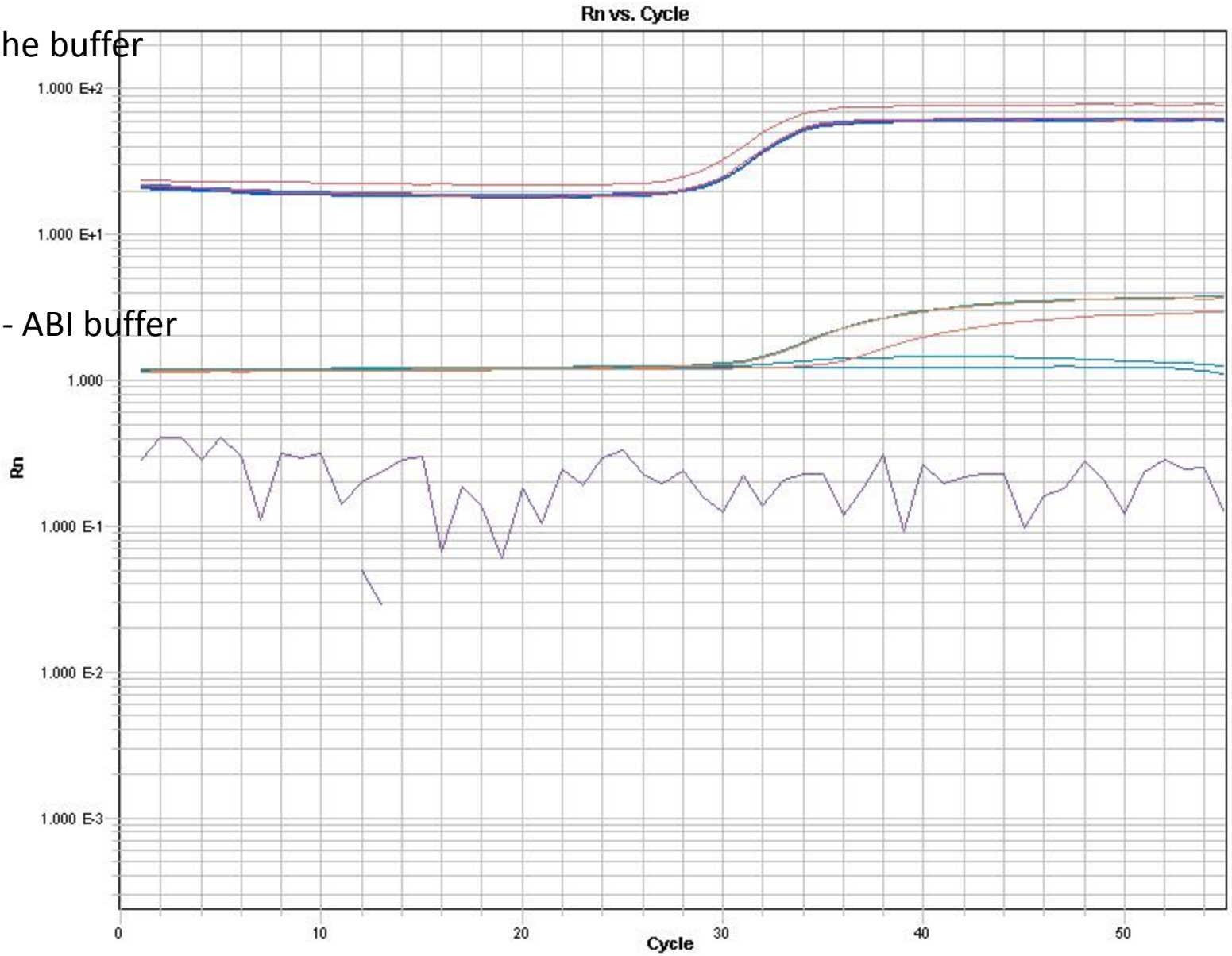
VIM RT1 (methyl) at 68 degrees in ABI machine (pos control samples, allidentical repeats in different wells), Roche buffer is earlier Ct and 2 Log Higher

TOP- Roche buffer

BOTTOM- ABI buffer



VIM RT29 (unmethyl) at 64 degrees in ABI machine (pos control samples, all Identical in different wells) , Roche buffer is earlier Ct and 2 Log Higher

TOP- Roche buffer

BOTTOM- ABI buffer